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TITLE: Therapeutic Targeting of TRPV1 for the Treatment of Chronic Pain  
Associated with Prostate Cancer Bone Metastasis

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14. ABSTRACT Prostate cancer (PCa) is the most common cause of cancer in men, with advanced form of this cancer frequently leads to bone metastasis, resulting in moderate to severe chronic pain in the back, pelvis and hips, thereby affecting the survivorship and quality of life in these patients. However, the currently available analgesics do not provide effective pain management under these pathological conditions. We proposed to study the precise cellular and molecular mechanisms that underlie nociceptor sensitization and pain sensation associated with bone-metastasized PCa. Specifically, our study is aimed at determining the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6 and TNF- $\alpha$ , PTHrP and ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization. Further, to test this hypothesis <i>in vivo</i> , we proposed to determine the role of IL-6/TNF- $\alpha$ /PTHrP/ET-1 in mediating pain sensitization in <i>scid</i> mice with xenografts of human PCa cells that metastasize to bones. By far our results show that IL-6/TNF- $\alpha$ /PTHrP/ET-1 sensitize TRPV1 channel activity in sensory neurons that innervate bones. Our results also show that in the presence of IL-6/TNF- $\alpha$ /PTHrP/ET-1, the TRPV1 channel could be activated at mild acidic pH conditions that are hallmarks of metastatic bone tumor microenvironment. Such modulations and mild acid activation of TRPV1 could lead to constitutive sensory neurons firings; thereby provide a mechanism for chronic pain associated with bone-metastasized PCa. Our results on bone-related pain behavior assessments in <i>scid</i> mouse xenografts of human PCa cells, 22Rv1-luc cells, identified several chronic and un-evoked pain behaviors in these mice, specific to bone-metastasized tumor growth.					
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## INTRODUCTION:

Prostate cancer (PCa) is the most common cause of cancer in men, with 60-80% subjects susceptible to bone metastasis and subsequent tumor growth of androgen-independent PCa cells. Bone metastasized PCa tumor growth leads to moderate to severe chronic pain in the back, pelvis and hips, due to a combination of tumor destruction of bones and constriction of nearby nerves or the spinal cord, which severely impacts the survivorship and quality of life (QoL) of these patients. Thus, pain relief is a key therapeutic goal to improve the QoL of men suffering from advanced PCa. Unfortunately, the currently available analgesics, such as opioid derivatives, bisphosphonates and non-steroidal anti-inflammatory drugs, do not provide adequate pain relief, due to lack of specificity and dose-limiting side-effects, which even include tumor growth-promoting effects, ultimately resulting in inadequate management of pain in patients with advanced bone-metastasized PCa. The major obstacle in the effective treatment of metastatic bone cancer pain has been our lack of understanding of the precise cellular/molecular mechanisms by which bone-metastasized PCa cells and the surrounding bone marrow microenvironment induce and maintain chronic pain. The overall goal of our study is to determine the precise cellular and molecular mechanisms that underlie nociceptor sensitization and pain sensation associated with bone-metastasized PCa. We are testing the hypothesis that inflammatory mediators and osteolytic/vasoactive peptides, such as interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), endothelin-1 (ET-1) and parathyroid hormone-related peptide (PTHrP), that are abundant in the metastatic bone tumor microenvironment, sensitize to adjacent sensory nerve fibers by modulation of the transient receptor potential vanilloid-1 (TRPV1) channel on these fibers. Our preliminary study found that sensory neurons that send nerve fibers to bones express the receptors for these mediators/peptides, and sustained activation of these receptors could lead to long-lasting upregulation of TRPV1 expression and function. Such modulations of TRPV1 would lead to constitutive channel activation and sensory neuron firing under patho-physiological conditions, a mechanism that presumably underlies the chronic pain sensation associated with bone metastasized PCa, without any overt stimulation. We proposed to test this hypothesis with two specific aims: 1) Determine the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6 and TNF- $\alpha$ , PTHrP and ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization. We proposed to employ patch-clamp electrophysiology, ratiometric  $\text{Ca}^{2+}$  imaging, and biochemical analysis of TRPV1 channel protein in cultured mouse dorsal root ganglia (DRG) neurons, as well as in DRG neurons co-cultured with 22Rv1 cells to address this question. 2) Determine IL-6-, TNF- $\alpha$ -, PTHrP- and ET-1-mediated pain sensitization in *scid* mice with xenografts of human prostate cancer cells that metastasize to bones. We proposed to utilize the human prostate cancer cell line 22Rv1-luc that stably express luciferase, which when grafted into severe combined immune-deficient (*scid*) mice metastasize to and grow tumors in bones with considerably frequency. Alongside monitoring bone metastasis/tumor-growth by bioluminescence imaging (BLI), we proposed to assess a battery of bone-related pain behaviors in these *scid* mouse xenografts of 22Rv1-luc PCa cells. Further, we proposed to utilize *in vivo* pharmacological blockade of TRPV1, and IL-6/TNF- $\alpha$ /PTHrP/ET-1 signaling, as well as to utilize 22Rv1-luc xenografts in *scid* mice lacking TRPV1, and subsequent assessments of bone-related pain behaviors to address this question. Our proposed study is aimed at identifying TRPV1 as a therapeutic target for the effective management of chronic pain associated with bone-metastasized PCa. Results from our studies utilizing mouse models of human PCa bone metastasis and chronic pain would serve as a precursor for the follow-up pre-clinical trial of small molecule antagonists of TRPV1 for treatment of metastatic bone cancer pain. With high incidences of bone-metastasized PCa in men in the US defense personnel, which significantly compromise their job efficiencies and QoL due to chronic pain, results from our study will accelerate the development of effective therapeutics for such pain.

## BODY:

Summarized below are the accomplishments from research work performed in the 2<sup>nd</sup> yr of this project.

**Milestone-1:** Determine the role of prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- $\alpha$ /PTHrP/ET-1 on upregulation of TRPV1 channel function/expression, and nociceptor sensitization (Aim 1).

**Major Goal/Objective 1:** Determine the precise modulation of TRPV1 channel activity in mouse sensory neurons by prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- $\alpha$ /PTHrP/ET-1.

**Task 1.** Acute sensitization of TRPV1 channel activity by IL-6/TNF- $\alpha$ /PTHrP/ET-1 (Aim 1.1; months 1-18). Primary cultures of DRG sensory neurons from adult mice, were used in these *in vitro* electrophysiological and Ca<sup>2+</sup> imaging studies. We also utilized the co-culture of 22Rv1-luc human PCa cells and RWPE-1 human prostate epithelial cells with mouse DRG neurons in patch-clamp electrophysiological and Ca<sup>2+</sup> imaging studies. We would like to reiterate that the tasks 1a and 1b were completed, and the tasks 1c and 1d were half-completed during the 1<sup>st</sup> year of the project.

1c. Determine the effects of IL-6/TNF- $\alpha$ /PTHrP/ET-1 on the temperature activation threshold of TRPV1 currents in mouse sensory neurons (months 8-14).

Accomplishments: We observed a significant decrease in the noxious temperature activation threshold of cultured mouse DRG neurons at a magnitude of 4°C to 7°C upon extracellular perfusion of IL-6, PTHrP and ET-1 (10 nM for each). However, extracellular perfusion of TNF- $\alpha$  (20 nM) did not lead to any significant decrease in the temperature activation threshold of cultured mouse DRG neurons. To verify that whether this drop was specific to the temperature activation threshold of TRPV1 similar experiments were carried out on cultured DRG neurons from *Trpv1*<sup>-/-</sup> mice, in which no significant temperature-activated inward currents were detected upto 48°C. Currently, we are continuing with similar experiments in mouse DRG neuron and 22Rv1-luc or RWPE-1 cell co-cultures, as well as in HEK293 cells expressing TRPV1 and the peptide receptors, IL-6R, PTH1R, TNFR2 and ET-1AR, in order to precisely determine the mechanism of decreased temperature-activation of the channel upon exposure to these peptide mediators.

1d. Determine the effects of IL-6/TNF- $\alpha$ /PTHrP/ET-1 on TRPV1 channel-mediated Ca<sup>2+</sup> influx in mouse sensory neurons by ratiometric functional Ca<sup>2+</sup> imaging (months 12-18).

Accomplishments: Although, these sets of experiments were proposed for the months 12 to 18, we already accomplished this task during the 1<sup>st</sup> year of the study. We have now expanded this study to verify the specificity of TRPV1 requirements, by performing Ca<sup>2+</sup> imaging experiments on cultured DRG neurons from *Trpv1*<sup>-/-</sup> mice, in which perfusion of IL-6, TNF- $\alpha$ , PTHrP (10 nM), and ET-1 (100 nM) did not lead to any significant cytosolic Ca<sup>2+</sup> elevation. These results conformed the sensitizing effects of these bone metastasis-specific bioactive peptides.

**Major Goal/Objective 2:** Determine the modulation of sensory neuron (nociceptor) firing by prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- $\alpha$ /PTHrP/ET-1.

**Task 1.** Acute sensitization of sensory neuron firing by IL-6/TNF- $\alpha$ /PTHrP/ET-1 (Aim 1.2; months 13-30). Primary cultures of DRG sensory neurons from adult mice were used in these *in vitro* current-clamp electrophysiological studies.

1a. Determine IL-6/TNF- $\alpha$ /PTHrP/ET-1-mediated sensitization of mouse sensory neuron firing in response to activation by capsaicin (months 13-18).

Accomplishments: We observed increased action potential (AP) firing in response to capsaicin application (50 nM, 30 sec), upon extracellular perfusion of PTHrP and ET-1 (10 nM each, 1 min) in between two capsaicin applications. Although some increase in the frequency of capsaicin-induced AP firing was observed in neurons upon extracellular perfusion of IL-6 and TNF- $\alpha$  (10 nM and 20 nM, respectively), they were not statistically significant. We would like to note here that such observations might result from differential levels of expression of IL-6R and TNFR2 in cultured mouse DRG neurons. Moreover, the number of neurons recorded from in these sets of experiments are 6-8, due to which we are now continuing with these experiments to increase the number of neurons for AP recordings to at least 15 for each treatment group. Additionally, we observed that PTHrP and ET-1 application (10 nM each) as such led to low-frequency AP firings with a time lag even without the application of capsaicin, which was absent in cultured DRG neurons from *Trpv1*<sup>-/-</sup> mice.

1b. Determine IL-6/TNF- $\alpha$ /PTHrP/ET-1-mediated sensitization of mouse sensory neuron firing in response to activation by acidic pH (months 19-24).

Accomplishments: Similar to our observations in capsaicin-evoked AP firings, we also observed increased AP firings in response to the application of pH6.4 extracellular buffer (30 sec), upon extracellular perfusion of PTHrP and ET-1 (10 nM each, 1 min) in between two pH6.4 applications, only in the subset of neurons that responded to capsaicin. Interestingly, extracellular perfusion of IL-6 and TNF- $\alpha$  (10 nM and 20 nM, respectively) also led to increased AP firings in response to extracellular pH6.4 buffer applications in capsaicin-responsive neurons. However, application of PTHrP, ET-1, IL-6 and TNF- $\alpha$  did not lead to any change in the frequency of pH6.4-induced AP firings in the subset of neurons that did not respond to capsaicin. These results suggested the key role of TRPV1 in upregulation of mild-to-moderate acidic pH-induced AP firing in sensory neurons, upon extracellular perfusion of IL-6/TNF- $\alpha$ / PTHrP/ET-1. To further substantiate these results we performed similar experiments in cultured DRG neurons from *Trpv1*<sup>-/-</sup> mice, in which no significant increase in pH6.4-induced AP firing was observed, in response to extracellular perfusion of IL-6/TNF- $\alpha$ / PTHrP/ET-1.

**Milestone-2:** Determine the role of upregulation of TRPV1 expression/activity mediated by prostate cancer bone metastasis-specific inflammatory factors, IL-6/TNF- $\alpha$ /PTHrP/ET-1, on development/sensitization of chronic bone-related pain, utilizing *scid* mouse xenografts of human prostate cancer cells that show bone metastasis; as well as pharmacological and genetic validation of TRPV1 as the key target for alleviating pain in the metastatic prostate/bone cancer-bearing mice (Aim 2).

**Major Goal/Objective 2:** Determine the role of IL-6, TNF- $\alpha$ , PTHrP and ET-1 in chronic pain associated with bone metastasis in *scid* mice with xenografts of 22Rv1 cells.

**Task 1.** Role of tumor microenvironment-derived IL-6, TNF- $\alpha$ , PTHrP and ET-1 in the development of bone pain behavior of *scid* mice with 22Rv1 xenografts (Aim 2.2; months 13-19).

1a. Generation of 22Rv1-luc xenografts in *scid* mice, weekly monitoring of tumor metastasis/growth by BLI, and weekly bone-related pain behavior assessments, before and after systemic injection of cocktail of neutralizing antibodies against IL-6/TNF- $\alpha$ /PTHrP/ET-1 (months 13-15).

Accomplishments: We generated xenografts of human PCa cells, 22Rv1-luc, and monitored tumor growth and bone metastasis by BLI on a weekly basis for up to 7 weeks after intracardiac cancer cell injections. Alongside, we made assessment of a battery of bone-related pain behaviors, such as thermal hyperalgesia, mechanical hypersensitivity, gait analysis, grip-strength and relative weight bearing. We are currently in the process of continuing with similar xenograft/pain assessment experiments with the injection of IL-6/TNF- $\alpha$ /PTHrP/ET-1 neutralizing antibodies. First, we are verifying the specificity of these neutralizing antibodies in a series of *in vitro* experiments with the use of DRG neuron and cancer cell co-cultures.

1b. Histopathological and radiological analyses of tumor growth and bone destruction, as well as analysis of TRPV1 expression in DRG in mouse xenografts mentioned above in the sub-task (1a) (months 15-19).

Accomplishments: After the behavioral assessments of bone-related pain behaviors in the *scid* mouse xenografts on the 7<sup>th</sup> week post cell injection, we euthanized these animals and performed ex vivo analysis of tumor growth using BLI, followed by radiological analysis of bone destruction. All the *scid* mice xenografts of 22Rv1-luc human PCa cells that exhibited metastatic tumor growth in hind limb bones showed considerable bone destruction, although no fractures were observed. Further, we performed whole animal perfusion with fixative, and subsequently removed the femur and tibia-fibula for tissue sectioning and H&E, as well as immunostaining for nerve fiber sprouting. Additionally, we removed both the pairs of L4-L6 DRGs (ipsi- and contra-lateral to metastatic tumor growth) from tumor-bearing and non-tumor mice for subsequent sectioning and immunostaining. Currently, both these tissue section stainings are underway, which we plan to complete in the next three months.

1c. Thorough analyses of metastatic bone tumor-induced chronic pain behavior in mouse xenografts (months 15-19).

Accomplishments: The human PCa cell, 22Rv1-luc, injected mice, which subsequently developed metastatic tumors in the hind limb bone showed increased peripheral pain behaviors such as thermal hyperalgesia (determined by paw withdrawal latency to light beam with increased temperature to the paws), and mechanical allodynia and hyperalgesia in a progressive manner, starting 5 weeks after cancer cell injections. Figure 1 shows exemplary decrease in the paw withdrawal latency after 6 weeks, in mice with tumors growing in the hindlimbs, as compared to mice with non-limb tumors. These changes were observed starting 5 weeks post cancer cell-injections. We also observed alterations in grip-strength, relative weight-bearing and several gait parameters in these mice with metastatic hindlimb tumors, progressively starting 4 weeks after cancer cell injections. Figure 2 shows exemplary alterations in four representative gait parameters after 4 and 6 weeks, relative to baseline values, in mice with tumors growing in the hindlimbs, as compared to mice with non-limb tumors. At present we are continuing with couple of more cohorts of mice for similar experiments, and the end of all the experiments data from >8 animals in each group will be pulled to prepare the plots and statistical analysis of the data.

**Major Goal/Objective 3:** Determine the role of TRPV1 in chronic pain associated with bone metastasis in *scid* mice with xenografts of 22Rv1 cells, by utilizing both *in vivo* pharmacological and genetic approach.

**Task 1.** Role of TRPV1 in bone pain in 22Rv1-luc *scid* mouse xenografts (Aim 2.3; months 20-36).

1a. Generation of 22Rv1-luc xenografts in *scid* mice, weekly monitoring of tumor metastasis/growth by BLI, and weekly bone-related pain behavior assessments, before and after systemic injection of highly-specific small molecule antagonist of TRPV1 (months 20-23).

Accomplishments: We generated xenografts of human PCa cells, 22Rv1-luc, and monitored tumor growth and bone metastasis by BLI on a weekly basis for up to 7 weeks after intracardiac cancer cell injections. Alongside, we made assessment of a battery of bone-related pain behaviors, such as thermal hyperalgesia, mechanical hypersensitivity, gait analysis, grip-strength and relative weight bearing. Starting week-3 after cell injections the animals were injected with TRPV1 small molecule antagonist (AMG9810, i.p., 30 mg/KG body weight) once a week and complete sets of pain-related behaviors were assessed. Twenty-four hr before TRPV1 antagonist injection, 1<sup>st</sup> set of behavioral assessments were performed, in order to compare the effects of TRPV1 antagonists on a weekly basis.

1c. Histopathological and radiological analyses of tumor growth and bone destruction, as well as analysis of TRPV1 expression in DRG in mouse xenografts mentioned above in the sub-task (1a) (months 23-25).

Accomplishments: After the behavioral assessments of bone-related pain behaviors in the *scid* mouse xenografts on the 7<sup>th</sup> week post cell injection, we euthanized these animals and performed ex vivo analysis of tumor growth using BLI, followed by radiological analysis of bone destruction. All the *scid* mice xenografts of 22Rv1-luc human PCa cells that exhibited metastatic tumor growth in hind limb bones showed considerable bone destruction, although no fractures were observed. Further, we performed whole animal perfusion with fixative, and subsequently removed the femur and tibia-fibula for tissue sectioning and H&E, as well as immunostaining for nerve fiber sprouting. Additionally, we removed both the pairs of L4-L6 DRGs (ipsi- and contra-lateral to metastatic tumor growth) from tumor-bearing and non-tumor mice for subsequent sectioning and immunostaining. Currently, both these tissue section stainings are underway, which we plan to complete in the next three months.

1d. Thorough analyses of metastatic bone tumor-induced chronic pain behavior in these (1a) mouse xenografts (months 23-25).

**Accomplishments:** The human PCa cell, 22Rv1-luc, injected mice, which subsequently developed metastatic tumors in the hind limb bone showed increased thermal and mechanical hyperalgesia, as well as alterations in grip-strength, relative weight-bearing and several gait parameters in a progressive manner, starting 4 weeks after cancer cell injections. Comparing the results from AMG9810-injected animals before and after drug injection from that specific duration of tumor growth, we found that most of the changes in pain-related behaviors due to metastatic PCa/bone tumor growth were attenuated by this TRPV1 antagonist (Figures 1&2). We are still continuing with completing these analyses, as well as starting another cohorts of mice for similar experiments. At the end of these experiments data from >8 animals in each group will be pulled to prepare the plots and statistical analysis of the data.

## KEY RESEARCH ACCOMPLISHMENTS:

- During the second year of this project we now confirmed that IL-6, TNF- $\alpha$ , PTHrP and ET-1, all of which are found elevated in the PCa/bone tumor microenvironment, induce nociceptor sensitization by specifically upregulating TRPV1 channel activity. Specifically, our results show that TRPV1, which under physiological conditions is minimally activated at mild-to-moderate acidic pH (example - pH6.5), can be robustly activated at such pH range upon exposure to IL-6/TNF- $\alpha$ /PTHrP/ET-1. Since the metastatic bone tumor microenvironment is characterized by such acidic pH conditions, our results implicate a constitutive activation of TRPV1 and nociceptor sensitization under such circumstance. To this end, our study also found that induction of proton-activated nociceptor firing in mouse DRG neurons in response to the mediators are specific to TRPV1 channel, since such modulatory effects were absent in DRG neurons from *Trpv1*<sup>-/-</sup> mice.
- Our results also show that upon exposure to IL-6/TNF- $\alpha$ /PTHrP/ET-1 the temperature activation threshold of TRPV1 is shifted to <37°C, which under physiological conditions is activated at noxious temperatures ( $\geq 43^\circ\text{C}$ ). These results suggested constitutive activation of TRPV1 channel on sensory nerve fibers adjacent to PCa/bone tumor microenvironment. Such modulatory actions on TRPV1 and nociceptor firing provide a cellular/molecular mechanism underlying chronic pain associated with bone-metastasized PCa.
- Our results showed development of thermal and mechanical hypersensitivities, as well as alterations in hindlimb weight-bearing, hindlimb grip-strength and several gait parameters in *scid* mouse xenografts of human PCa cells, 22Rv1-luc.
- Our results from in vivo pharmacological experiments with the systemic injection of a specific small molecule antagonist of TRPV1, AMG9810, show attenuation of majority of these pain-related behaviors in *scid* mouse xenografts of 22Rv1-luc human PCa cells that specifically developed metastatic bone tumors in the hindlimbs..

## REPORTABLE OUTCOMES:

### Manuscripts:

Currently, we are preparing a manuscript comprising of our results on TRPV1 channel modulation and nociceptor sensitization by inflammatory mediator and vasoactive peptides that are secreted at elevated levels in the metastatic PCa/bone tumor microenvironment.

### Scientific Presentations:

I presented a platform presentation covering our results so far on TRPV1 channel modulation and nociceptor sensitization by PCa/bone tumor environment-specific inflammatory mediators and vasoactive peptides in the Annual meeting of the American Pain Society. Also, a portion of these results, specifically on the intracellular signaling cascades underlying PTHrP-modulation of TRPV1, were presented in the annual meeting of the Society for Neuroscience, October 2012 as a scientific poster presentation.



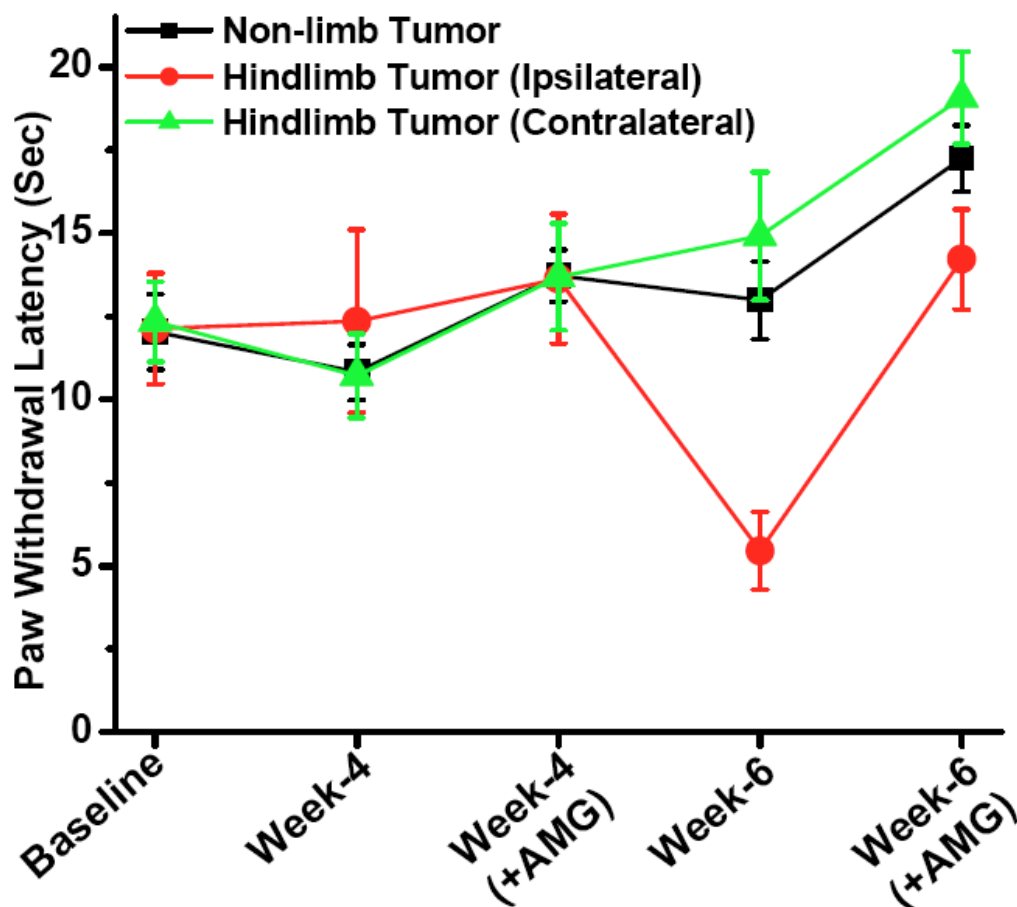
## CONCLUSION:

In conclusion, our results and observations from the 2<sup>nd</sup> year of this study have led us to suggest that inflammatory mediators and osteolytic/vasoactive peptides, IL-6, TNF- $\alpha$ , PTHrP and ET-1, that are secreted at elevated levels in the metastatic PCa/bone tumor microenvironment sensitize nociceptor firing via TRPV1 channel. Specifically, our results show that TRPV1, which under physiological conditions is minimally activated at mild-to-moderate acidic pH (example - pH6.5), can be robustly activated at such pH range upon exposure to IL-6/TNF- $\alpha$ /PTHrP/ET-1. Since the metastatic bone tumor microenvironment is characterized by such acidic pH conditions, our results implicate a constitutive activation of TRPV1 and nociceptor sensitization under such circumstance. In addition, our results also show that physiologically TRPV1 on sensory neurons is activated at noxious temperatures,  $\geq 43^{\circ}\text{C}$ ; however, upon exposure to IL-6/TNF- $\alpha$ /PTHrP/ET-1 the temperature activation threshold of TRPV1 is shifted to  $< 37^{\circ}\text{C}$ , suggesting constitutive activation of TRPV1 channel on sensory nerve fibers adjacent to PCa/bone tumor microenvironment. These molecular modulatory actions on TRPV1 and nociceptor firing provide a cellular/molecular mechanism underlying chronic pain associated with bone-metastasized PCa. Further, our *in vivo* results pertaining to bone-related pain behavior assessments in *scid* mouse xenografts of human PCa cells, 22Rv1-luc, determined the development of thermal and mechanical hypersensitivities, as well as alterations in hindlimb weight-bearing, hindlimb grip-strength and several gait parameters. Systemic injection of a specific small molecule antagonist of TRPV1 (AMG9810) led to the attenuation of majority of these pain-related behaviors in *scid* mouse xenografts of 22Rv1-luc human PCa cells that specifically developed metastatic bone tumors in the hindlimbs. Currently, we are continuing with our *in vitro* studies to determine the mechanistic bases for metastatic PCa bone tumor-induced upregulation of TRPV1 expression and sensory neuron firing, as well as our *in vivo* studies utilizing *in vivo* pharmacological and genetic approaches, in order to determine the role of TRPV1 in chronic bone-related pain behaviors in *scid* mouse xenografts of human PCa cells.

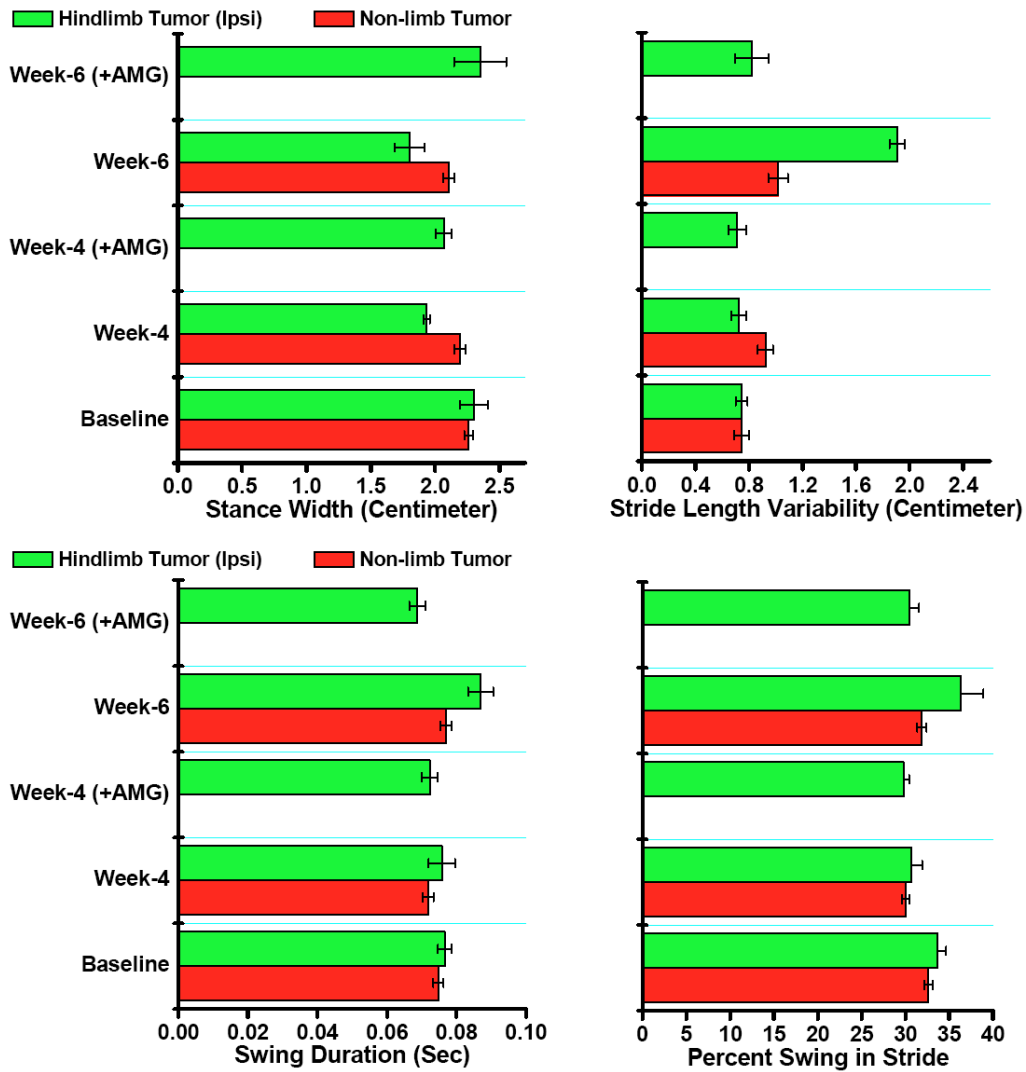
**REFERENCES:** None

**APPENDICES:** None

# SUPPORTING DATA:



**Figure 1.** Development of thermal hyperalgesia in response to bone-metastasized PCa tumor growth in *scid* mouse xenografts of 22Rv1-luc cells, and its attenuation by the TRPV1 antagonist AMG9810. Hargreaves method of paw withdrawal latency (PWL in second) determination in response to a light beam on increased temperature at the hind paws was performed. Decrease in this latency suggests the development of thermal hyperalgesia. *Scid* mouse xenografts of human PCa 22Rv1-luc cells were used in this experiment. Baseline measurement of PWL was performed one day before intracardiac injection of 22Rv1-luc cells, and subsequent PWL measurements were performed on a weekly basis. Animals exhibiting metastatic hindlimb tumors were grouped as “Hindlimb Tumor” group, with separate determination of PWLs in the ipsilateral (tumor-bearing) and contralateral (non-tumor) limbs. Animals with tumor growth in regions other than hindlimb were grouped as the “Non-limb Tumor”. No visible change were observed in the PWL of Non-limb Tumor group (n=6), as well as in the contralateral hindlimbs of Hindlimb Tumor group (n=4), at weeks 4 and 6 after cancer cell injections. A drastic reduction was observed in the PWL of ipsilateral hindlimbs of the Hindlimb Tumor group (n=4), at week-6, but not at week-4 after cancer cell injections. Such reduction in the PWL was attenuated by systemic injection of the specific TRPV1 antagonist, AMG9810 (i.p., 30 mg/KG body weight; n=4). Data are presented as mean  $\pm$  standard error of mean (SEM).



**Figure 2. Alterations in gait parameters in response to bone-metastasized PCa tumor growth in *scid* mouse xenografts of 22Rv1-luc cells, and its attenuation by the TRPV1 antagonist AMG9810.** Digital gait analysis method was performed, as detailed in the approach section of the project. Twentyfive different gait parameters were determined, however, only four (stance width, stride length variability, swing duration, and percent swing in stride) of the 7 parameters, where changes were observed, are shown here. *Scid* mouse xenografts of human PCa 22Rv1-luc cells were used in this experiment. Baseline measurement of gait was performed one day before intracardiac injection of 22Rv1-luc cells, and subsequent gait measurements were performed on a weekly basis. Animals exhibiting metastatic hindlimb tumors were grouped as “Hindlimb Tumor” group, with separate determination of gait parameters in the ipsilateral (tumor-bearing) and contralateral (non-tumor) limbs. Animals with tumor growth in regions other than hindlimb were grouped as the “Non-limb Tumor”. No visible change were observed in these gait parameters of Non-limb Tumor group (n=6), as well as in the contralateral hindlimbs of Hindlimb Tumor group (not shown), at weeks 4 and 6 after cancer cell injections. Decreased stance width was observed in the ipsilateral hindlimbs of the Hindlimb Tumor group (n=5), at weeks 4 and 6 after cancer cell injections. However, increased in swing durations, stride length variability and percent swing in stride were observed in the ipsilateral hindlimbs of the Hindlimb Tumor group (n=5), only at week-6 after cancer cell injections. Such alterations in the above-mentioned gait parameters were attenuated by systemic injection of the specific TRPV1 antagonist, AMG9810 (i.p., 30 mg/KG body weight; n=5). Data are presented as mean ± SEM.